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Running title: Variability in tench by mtDNA analysis

Genetic variability in tench (*Tinca tinca* L.) as revealed by PCR-RFLP analysis of mitochondrial DNA

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Abstract

Four mitochondrial DNA segments, ND1, ND6, cyt *b* and D-loop, were analysed by polymerase chain reaction-restriction fragment lenght polymorphism (PCR-RFLP) in 14 tench (*Tinca tinca* L.) populations located in Europe and Asia; also data on five Italian populations previously analysed for the same mtDNA segments were included in the study. All the considered segments were polymorphic and originated a total of 9

composite haplotypes, which were clustered into two haplogroups, A and B, possibly corresponding to the Western and Eastern phylogroups previously described in tench. Nine out of 19 populations showed polymorphism, with haplotype diversity ranging from 0.246 to 0.643 and nucleotide diversity from 0.009 to 0.078. Seventy-five percent of the pairwise comparisons were significant, indicating a high between-population variability. The Neighbour-Joining tree revealed the presence of three clusters, including 'pure' populations, with only A or B haplogroup, and 'mixed' populations, with both haplogroups. The possibility of identifying populations with different haplotypes has practical implications for both conservation and supportive stocking.

Key words: *Tinca tinca*, Mitochondrial DNA, RFLP, Genetic variability.

Introduction

For the last twenty years the genetic research in aquaculture has been exponentially increasing, but genetic information on tench (*Tinca tinca* L.) is still limited compared to other fish species. In fact, apart from some studies carried out in the past decades by means of protein markers (Valenta *et al.*, 1978; Šlechtova *et al*., 1995; Kohlmann & Kersten, 1998), only recently tench specific microsatellite loci have been described (Kohlmann & Kersten, 2006) and used to characterize many European and Asian populations (Kohlmann *et al*., 2007, 2010; Lo Presti *et al*., 2010b). Also, the complete mitochondrial DNA (mtDNA) sequence has been made available only recently (Saitoh *et al*., 2006) and the analysis of the mitochondrial cyt *b* gene, together with three nuclear genes, contributed to elucidate the molecular phylogeography of the tench, with

the discovery of two geographical clades (Eastern and Western), possibly developed in response to recurrent isolation in glacial refugia during the Pleistocene (Lajbner *et al*., 2007). Within the Eastern phylogroup, the analysis of cyt *b* also allowed to identify populations distinct from the major Eastern clade in the Anzalee lagoon of the Caspian Sea in Iran and in the Iskar River of the Danube River drainage in Bulgaria (Lajbner *et al*., 2011). Only a single study was devoted to analyse the polymorphism of different mitochondrial segments as a tool to detect the tench genetic variability (Lo Presti *et al*., 2010a).

The aim of this paper is to extend the study of the mtDNA polymorphisms to a larger number of tench populations distributed in a wide geographical area, in order to get a more comprehensive picture of the within- and between-population variability in tench.

Materials and methods

A total of 126 individuals were analysed, belonging to 14 wild and cultured populations, located in different European and Asian countries; also the data on the five Italian populations previously studied for the same mtDNA segments (Lo Presti *et al*., 2010a) were included, in order to cover a larger geographical area (Table 1). All the populations had been already analysed by microsatellite markers (Kohlmann *et al*., 2010; Lo Presti *et al*., 2010b).

Total genomic DNA was extracted from muscle or fin using the NucleoSpin Tissue kit (Macheray-Nagel, Düren, Germany). PCR reactions to amplify ND1, ND6, cyt *b* and D-loop segments were performed as described in Lo Presti *et al*. (2010a). Each amplicon was digested with 4 restriction enzymes, which were selected on the basis of the previous results (Lo Presti *et al*., 2010a) and considering the expected restriction

pattern, derived by virtually digesting the reference sequence with Webcutter 2.0 (Heiman, 1997). Some of the enzymes were used to digest different amplicons (Table 2), so that a total of seven endonucleases were employed: *Alu*I, *Sau3A*I (Sigma, St Louis, MO, USA), *Ase*I, *Hae*III, *Msp*I (New England BioLabs, Beverly, MA, USA) *Hind*III, *Hinf*I (Fermentas, Burlington, ON, Canada). The digested fragments were resolved on 2% agarose gels, stained with ethidium bromide and visualized under UV light. The size of the fragments was estimated in comparison with a 100 bp size ladder (Sigma, St Louis, MO, USA) and each different pattern produced by each enzyme was identified by a single letter code, with A assigned to the pattern expected on the basis of the reference sequence. Composite haplotypes were designed by a 16-letter code, representing the pattern for each restriction enzyme.

The relationships between composite haplotypes were analysed by calculating the mean number of substitutions per site between all pairs of haplotypes from restriction site data (Nei & Li, 1979), which were used to construct a Neighbour-Joining tree as implemented in PHYLIP ver 3.5 package (Felsenstein, 1993); the reliability of the tree topology was tested by 1,000 bootstrap replicates.

ARLEQUIN ver. 3.1 program (Excoffier *et al*., 2005) was used to evaluate the variability within populations by haplotype and nucleotide diversity (Nei & Tajima, 1981), as well as to test the population differentiation by the pairwise exact test (Raymond & Rousset, 1995). Significance levels for multiple comparisons were adjusted using the sequential Bonferroni correction (Rice, 1989). The genetic distances between populations were also estimated as the pairwise net nucleotide divergence (Nei & Li, 1979), followed by the construction of the Neighbour-Joining tree, using the MEGA 4 software (Tamura *et al*., 2007).

Results

All the enzymes but *Msp*I detected restriction fragment length polymorphisms at some mtDNA segment (Table 2). The digestion of ND6 and ND1 revealed one and two variants, respectively, as previously reported (Lo Presti *et al*., 2010a), while additional variation was observed for cyt *b* and D-loop. At cyt *b* the *Sau3A*I endonuclease detected a new variant, whose pattern does not seem to derive directly from the loss or gain of a restriction site with respect to the reference pattern, so that a more complex situation could be hypothesized, such as concomitant loss and gain of restriction sites. For the D-loop two new variants were found, one with *Ase*I and one with *Hae*III, respectively due to the presence and absence of a restriction site.

The polymorphisms at the four mtDNA segments originated a total of nine composite haplotypes, named H1 to H9, with H1 corresponding to the reference sequence (Table 3). The analysis of the overall frequencies indicated that H1 and H2 were the most frequent composite haplotypes, with a cumulative frequency of 0.805, while the others were very rare, with frequencies lower than 0.05.

The analysis of the pairwise nucleotide divergence between composite haplotypes led to a phylogenetic tree where two highly divergent haplogroups were identified: one, designated as haplogroup A, included the H1, H3, H4, H5 and H6 composite haplotypes, while the other, designated as haplogroup B, included the H2, H7, H8 and H9 composite haplotypes (Fig. 1). The two haplogroups differed for polymorphisms at the ND1 and Cyt *b* segments: all the haplotypes belonging to the haplogroup A had the restriction morphs ND1/*Alu*I A, ND1/*Hinf*I A and Cyt *b*/*Sau3A*I A, while all the haplotypes of the haplogroup B had the restriction morphs B at the same sites (Table 3).

121 The bootstrap value of 96% strongly supported the between-haplogroup differentiation,
122 while the within-haplogroup relationships were less clear, with low to medium bootstrap
123 values.
124 As for the composite haplotype distribution, H1 and H2 were present in 63% and 47%
125 of the populations, respectively, whereas the others were limited to one or few
126 populations (Table 4). H3 was observed only in the Central and Southern Italian
127 populations (BOL, TRA, ALC); H4 and H6 were the rarest composite haplotypes, found
128 in one individual only from Trasimeno (Italy) and Felchowsee (Germany) lakes,
129 respectively. H5, H7 and H8 were private haplotypes for the wild VAL and TUR, and
130 cultured MAL populations, respectively. Moreover, H8 seemed to be fixed in the latter
131 population, so it might be used as a genetic tag, if the data would be confirmed on a
132 larger sample (the present sample size is 10 individuals only). H9 was fixed in the GOL
133 population (the golden colour variety), but present also in one wild FEL individual.
134 Ten out of 19 populations exhibited no variability (Table 4). For ISE and BRA the
135 finding is possibly dependent on the low sample size (three and four individuals,
136 respectively), and therefore these two Italian wild populations were excluded from the
137 subsequent analysis. On the contrary, the fixation of the haplotype H9 in GOL can be
138 interpreted as a result of the founder effect (Kvasnika *et al*., 1993). It is worth to
139 underline that the other monomorphic populations are cultured, except for the German
140 DÖL, which is wild. The absence of polymorphism in DÖL and KÖW is quite
141 unexpected, considering that these populations analysed by microsatellite markers
142 showed a high variability (Kohlmann *et al*., 2010).
143 In the polymorphic populations, the haplotype diversity ranged from 0.246 (PIA) to
144 0.643 (FEL), whereas the nucleotide diversity ranged from 0.009 (BOL) to 0.078 (FEL)

(Table 4). As expected, the highest values for nucleotide diversity were observed in the populations where composite haplotypes of both evolutionary lineages were present. In particular, FEL showed the highest values for both indices, confirming its importance as a reservoir of genetic diversity, in agreement with the high variability detected by previous studies on microsatellite markers (Kohlmann *et al*., 2010).

Concerning the between-population differentiation, 75% of the pairwise comparisons were significant, indicating a high level of genetic variation at species level (Table 5). Going into more detail, GOL, MAL and VAL statistically differed from all the other populations, while most of the nonsignificant comparisons involved the Eastern populations (CHI, TUR, HUN, ROM, VEM and VOD) and the one from Spain (BAD). No differences were observed between the German populations (FEL, KÖW, DÖL), or between those of Central-Southern Italy (TRA, BOL, ALC).

The Neighbour-Joining tree, constructed on the basis of the pairwise net nucleotide divergence, separated two clusters, one including the Italian and German populations and one including all the others (Fig. 2).

The latter displayed lack of resolution, involving populations all fixed for the H2 composite haplotype (BAD, CHI, HUN and VOD). These populations had a nucleotide divergence of 0.248, while the Italian and German populations represented a more heterogeneous group, with D_A of 0.711. The divergence between the two branches was much higher (D_A = 4.652), indicating a deep separation between the populations of the two groups. It is interesting to note that the populations with both haplogroups (FEL and PIA at one side, VEM and ROM at the other side) were located close to the principal node. Therefore, the tree can be subdivided into three clusters, corresponding to “pure” populations, with A or B haplogroup, and “mixed” populations, with both haplogroups.

Discussion

The PCR-RFLP analysis of ND1, ND6, cyt *b* and D-loop in 19 tench populations confirmed the effectiveness of these mtDNA markers for population genetic studies of this species. Of the four examined mtDNA segments, cyt *b* and D-loop showed the highest variability, with four and three variants, respectively. The quite high variability of the D-loop seems to be a peculiarity of the tench, not observed in other teleosts so far; for example no polymorphism was found in Danish brown trout (*Salmo trutta* L.) strains by digestion with 18 restriction enzymes (Hansen & Loeschke, 1996), nor in rainbow trout (*Onchorhyncuhus mykiss*) using 12 endonucleases (Sajedi *et al*., 2003). These findings underline that the mtDNA segments more appropriate for population studies have to be chosen for each species individually.

Nine out of 19 populations examined showed considerable haplotype as well as nucleotide diversity. However, the mtDNA markers generally revealed a lower power than microsatellite markers in detecting the within-population variability. In fact, the average haplotype diversity level of mtDNA (H_{mt}) observed in the present study (0.215) was lower than the average heterozygosity level of microsatellites (H_{ms}) deduced from the data of Kohlmann *et al*. (2010) and Lo Presti *et al*. (2010b) on the same populations (0.343). However, in some populations where mtDNA was polymorphic, H_{mt} was even higher than H_{ms} (TUR, FEL, ALC, TRA, BOL). The absence of relationships between nuclear and mitochondrial variability, already reported for other species (Palumbi & Baker, 1994), is not surprising, considering the different genetic background and mode of evolution of the two types of markers. Therefore, for the different information they

192 provide, the complementary analysis of nuclear and mitochondrial markers represent a
193 powerful strategy to elucidate the population genetic structure.
194 On the other hand, mtDNA markers proved to be an excellent tool in revealing the
195 between-population variability. The identification of two mtDNA haplogroups in the
196 present study as well as the recent discovery of two major growth hormone gene classes
197 in tench (Kocour & Kohlmann, 2011) further support the results of Lajbner *et al*. (2007,
198 2011), who investigated the molecular phylogeography of tench by the analysis of the
199 cyt *b* locus and some nuclear markers and evidenced that the species is subdivided into
200 deeply divergent Western and Eastern phylogroups, which are not distinct species
201 however (Lajbner *et al*., 2010). Also in other freshwater species, including the chub
202 (*Leuciscus cephalus*) (Durand *et al*., 1999), perch (*Perca fluviatilis*) (Nesbø *et al*., 1999)
203 and barbel (*Barbus barbus*) (Kotlik & Berrebi, 2001), genetic lines related to the
204 geographical location were observed, which could indicate an evolutionary history
205 common to different freshwater species.
206 On the basis of the haplogroup composition, composite haplotype distribution and
207 population location, it can be inferred that the haplogroup A observed in the present
208 study corresponds to the Western phylogroup reported by Lajbner *et al*. (2007, 2011),
209 while the haplogroup B corresponds to the Eastern phylogroup.
210 It is noteworthy, for practical implications, that the mtDNA markers used in the present
211 study allowed a good resolution for both phylogroups, compared to the markers used by
212 Lajbner *et al*. (2011) and Lajbner and Kotlik (2011), that detected three subclades in the
213 Eastern clade but showed only very little internal structure for the Western clade.
214 The Neighbour-Joining tree constructed on the basis of the pairwise net nucleotide
215 divergence (Nei & Li, 1979) between populations showed some similarities with the

tree obtained by Kohlmann *et al*. (2010) using the microsatellite markers: in both cases HUN, CHI, BAD and TUR clustered together and the German populations were located in the opposite branch. In particular, the results confirmed the genetic similarity between the Spanish and Chinese populations, which could be explained by human introduction of tench from East to Spain.

Conclusions

The present PCR-RFLP based analysis of four segments of the tench mtDNA revealed considerable haplotype as well as nucleotide diversity in nine out of 19 populations examined. Thus, these easily and inexpensively to screen polymorphisms might effectively be used for population genetic studies of this species, with implications for conservation and supportive stocking. For conservation purposes, these mtDNA markers might help to identify populations with different haplotypes within haplogoups and could thus contribute to protect their genetic integrity. In case of stocking, donor and recipient populations should genetically be similar as much as possible, i.e. they should at least belong to the same composite haplotype. On the other hand, mixed populations with higher mtDNA diversity (and also higher microsatellite variability) might be valuable baseline populations to start selective breeding programs, in particular if mtDNA and microsatellite information would be combined with the recently described tench growth hormone gene polymorphisms.

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314

315 Table 1. Description of the populations.

Population	Code	n.	Status	Geographical location
Alcantara [(1)]	ALC	5	Wild	Alcantara river, Italy
Badajoz	BAD	10	Cultured	Badajoz, Spain
Bolsena [(1)]	BOL	21	Wild	Bolsena lake, Italy
Bracciano	BRA	4	Wild	Bracciano lake, Italy
China	CHI	10	Cultured	Wuhan, China
Döllnsee	DÖL	10	Wild	Döllnsee lake, Germany
Felchowsee	FEL	8	Wild	Felchowsee lake, Germany
Golden	GOL	10	Cultured	Colour variety developed in Vodñany, Czech Republic
Hungary	HUN	10	Cultured	Hungary, collected at Vodñany live gene bank
Iseo	ISE	3	Wild	Iseo lake, Italy
Königswartha	KÖW	10	Cultured	Königswartha, Germany
Marianske Lazne	MAL	10	Cultured	Czech Republic, year class 1998
Pianalto [(1)]	PIA	57	Cultured	Poirino highland, Italy
Romania	ROM	10	Cultured	Romania, collected at Vodñany live gene bank
Trasimeno [(1)]	TRA	9	Wild	Trasimeno lake, Italy
Turkey	TUR	11	Wild	Sapanca lake, Turkey
Valagola [(1)]	VAL	13	Wild	Valagola lake, Italy
Velke Mezirici	VEM	10	Cultured	Czech Republic
Vodñany 1998	VOD	10	Cultured	Czech Republic, year class 1998

316 [(1)]From Lo Presti *et al*., 2010a.

317

Table 2. Approximate fragment size of the restriction morphs observed by digesting four mtDNA segments with seven different endonucleases.

mtDNA segment	ND1						ND6				
Endonuclease	*Alu*I		*Hae*III	*Hinf*I		*Msp*I	*Hind*III		*Hinf*I	*Msp*I	*Sau3A*I
Restriction morph	A	B	A	A	B	A	A	B	A	A	A
Fragment size (bp)			964								
	570	570			383	496		576			
	317			328	328	346				316	361
				308	308		310		287		
		185		226			266		275	260	215
		132		157		177					
	74	74									
	58	58	55								
									14		

Table 2. Continued.

mtDNA segment	Cytb								D-loop						
Endonuclease	*Alu*I			*Hae*III		*Hinf*I	*Sau3A*I		*Alu*I		*Ase*I		*Hae*III		*Hinf*I
Restriction morph	A	B	C	A	B	A	A	B	A	B	A	B	A	B	A
Fragment size (bp)	985		1146	1146	1059										
		707				495			634	634	758	659	596	596	568
						480	305	320						402	
		278					297	297							375
							224	234	240				270		
	161	161					213	225		~220	119	119			
						129						99	132		
					87			70	90	90	86	86			
						42			34	34	35	35			55
										~20					

329

330 Table 3. Composite haplotypes and their overall frequency (p).

Haplotype	ND1	ND6	cyt b	D-loop	p
H1	AAAA	AAAA	AAAA	AAAA	0.498
H2	BABA	BAAA	BAAB	AAAA	0.307
H3	AAAA	AAAA	AAAA	BAAA	0.039
H4	AAAA	AAAA	ABAA	BAAA	0.004
H5	AAAA	AAAA	CAAA	AAAA	0.039
H6	AAAA	AAAA	AAAA	AABA	0.004
H7	BABA	BAAA	BAAB	AABA	0.017
H8	BABA	BAAA	BAAB	ABAA	0.043
H9	BABA	AAAA	BAAB	ABAA	0.048

331

332

333 Table 4. Within-population variability: haplotype frequency, haplotype (H) and nucleotide (π) diversity (mean value ± standard error).

POP	H1	H2	H3	H4	H5	H6	H7	H8	H9	H ± s.e.	π ± s.e.
BAD	-	1.000	-	-	-	-	-	-	-	-	-
CHI	-	1.000	-	-	-	-	-	-	-	-	-
TUR	-	0.636	-	-	-	-	0.364	-	-	0.509 ± 0.101	0.014 ± 0.014
FEL	0.625	0.125	-	-	-	0.125	-	-	0.125	0.643 ± 0.184	0.078 ± 0.052
KÖW	1.000	-	-	-	-	-	-	-	-	-	-
DÖL	1.000	-	-	-	-	-	-	-	-	-	-
ROM	0.200	0.800	-	-	-	-	-	-	-	0.356 ± 0.159	0.058 ± 0.040
HUN	-	1.000	-	-	-	-	-	-	-	-	-
GOL	-	-	-	-	-	-	-	-	1.000	-	-
MAL	-	-	-	-	-	-	-	1.000	-	-	-
VEM	0.300	0.700	-	-	-	-	-	-	-	0.467 ± 0.132	0.076 ± 0.049
VOD	-	1.000	-	-	-	-	-	-	-	-	-
ALC	0.400	-	0.600	-	-	-	-	-	-	0.600 ± 0.175	0.014 ± 0.015
BRA	1.000	-	-	-	-	-	-	-	-	-	-
BOL	0.762	-	0.238	-	-	-	-	-	-	0.381± 0.101	0.009 ± 0.010
ISE	1.000	-	-	-	-	-	-	-	-	-	-
PIA	0.860	0.140	-	-	-	-	-	-	-	0.246 ± 0.067	0.028 ± 0.020
TRA	0.778	-	0.111	0.111	-	-	-	-	-	0.417 ± 0.191	0.014 ± 0.014
VAL	0.308	-	-	-	0.692	-	-	-	-	0.462 ± 0.110	0.010 ± 0.011

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336 Table 5. Nucleotide divergence within population (diagonal) and between populations (above the diagonal); significance (*) of the exact test of

337 Raymond and Rousset (1995) for population differentiation (below the diagonal).

	BAD	CHI	TUR	FEL	KOW	DOE	HUN	ROM	GOL	MAL	VEM	VOD	PIA	VAL	BOL	TRA	ALC
BAD	*0.00*	0.00	0.11	3.43	6.00	6.00	0.00	0.13	2.00	1.00	0.40	0.00	4.42	6.46	6.05	6.03	6.30
CHI	.	*0.00*	0.11	3.43	6.00	6.00	0.00	0.13	2.00	1.00	0.40	0.00	4.42	6.46	6.05	6.03	6.30
TUR	.	.	*0.51*	3.45	6.11	6.11	0.11	0.24	2.11	1.11	0.51	0.11	4.53	6.57	6.16	6.14	6.41
FEL	*	*	*	*2.89*	0.18	0.18	3.43	1.71	3.43	4.18	1.05	3.43	0.10	0.64	0.23	0.21	0.48
KÖW	*	*	*	.	*0.00*	0.00	6.00	3.73	6.00	7.00	2.80	6.00	0.11	0.46	0.05	0.03	0.30
DÖL	*	*	*	.	.	*0.00*	6.00	3.73	6.00	7.00	2.80	6.00	0.11	0.46	0.05	0.03	0.30
HUN	.	.	.	*	*	*	*0.00*	0.13	2.00	1.00	0.40	0.00	4.42	6.46	6.05	6.03	6.30
ROM	.	.	.	*	*	*	.	*2.13*	1.73	1.13	-0.19	0.13	2.49	4.19	3.78	3.76	4.03
GOL	*	*	*	*	*	*	*	*	*0.00*	1.00	1.80	2.00	4.70	6.46	6.05	6.03	6.30
MAL	*	*	*	*	*	*	*	*	*	*0.00*	1.40	1.00	5.42	7.46	7.05	7.03	7.30
VEM	.	.	*	*	*	*	.	.	*	*	*2.80*	0.40	1.73	3.26	2.85	2.83	3.10
VOD	.	.	.	*	*	*	.	.	*	*	.	*0.00*	4.42	6.46	6.05	6.03	6.30
PIA	*	*	*	*	.	.	*	*	*	*	*	*	*1.47*	0.57	0.15	0.13	0.41
VAL	*	*	*	*	*	*	*	*	*	*	*	*	*	*0.46*	0.51	0.49	0.76
BOL	*	*	*	*	.	.	*	*	*	*	*	*	*	*	*0.38*	-0.03	0.06
TRA	*	*	*	.	.	.	*	*	*	*	*	*	*	*	.	*0.61*	0.06
ALC	*	*	*	.	*	*	*	*	*	*	*	*	*	*	.	.	*0.60*

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341 Fig. 1. Neighbor-Joining tree of the composite haplotypes. Only bootstrap values higher than 40% are shown.

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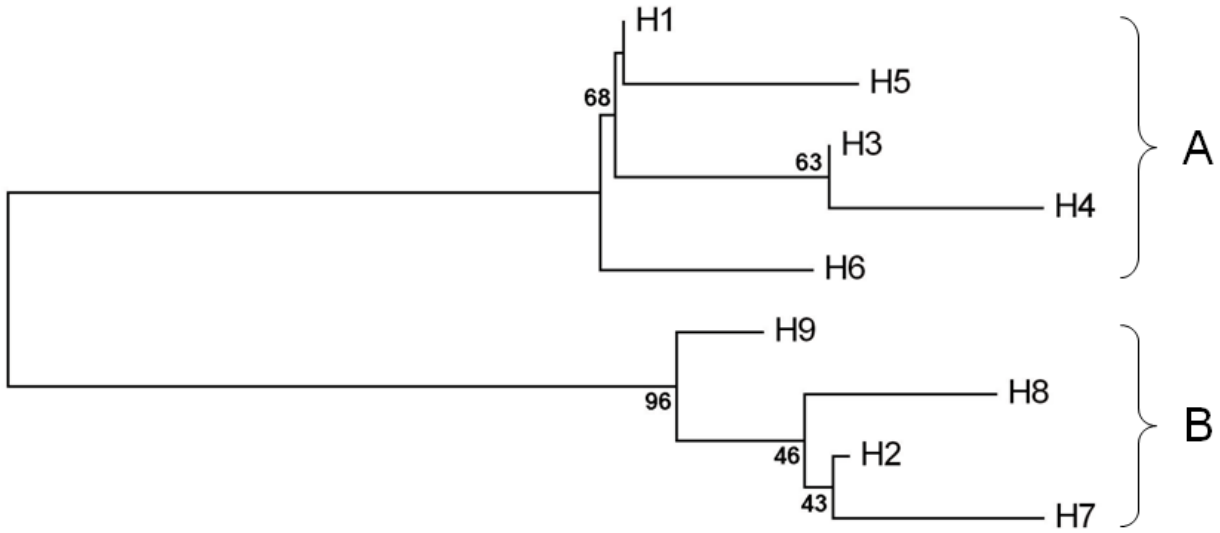

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347 Fig. 2. Neighbor-Joining tree of 19 tench populations.

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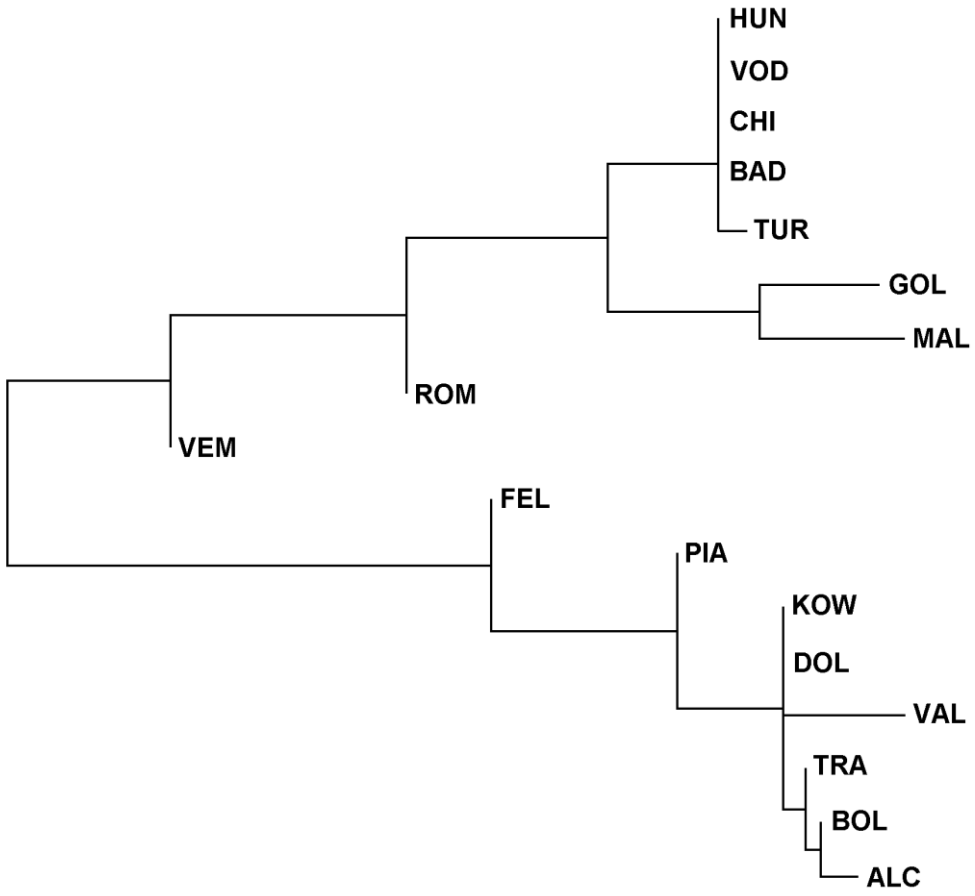

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